From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

BECKER-KURIG-STRAUS
Bavariastrasse 7
D-80336 München
ALLEMAGNE

BECKER KURIG STRAUS
BAVARIASTRASSE 7 · 80336 MÜNCHEN
12. Sep. 2001
WV: ..... /LF: .....

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	11.09.2001
Applicant's or agent's file reference 80295 WO(AS/LS)	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05403	09/06/2000	11/06/1999

Applicant
SOCIETE DES PRODUITS NESTLE S.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Hingel, W Tel.+49 89 2399-8717

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
80295 WO(AS/LS)		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05403	09/06/2000	11/06/1999
International Patent Classification (IPC) or national classification and IPC C12N13/00		
Applicant SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/12/2000	11.09.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer SCHEFFZYK, I Telephone No. +49 89 2399 8602

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/05403

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-12	as originally filed				

Claims, No.:

1-4	as received on	15/06/2001	with letter of	15/06/2001	

Drawings, sheets:

1/9-9/9	as originally filed				

Sequence listing part of the description, pages:

1-3, filed with the letter of 120900

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☒ furnished subsequently to this Authority in written form.

   ☒ furnished subsequently to this Authority in computer readable form.

   ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-3
	No:	Claims	4
Inventive step (IS)	Yes:	Claims	4
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-4
	No:	Claims	

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

SECTION V----------

Claims 1-3 are deemed novel and inventive since firstly a method for protecting L. johnsonii La1 against stress is not taught in the available prior art and secondly, although it is known in the prior art that certain microorganisms can be rendered insensitive against stress it is not predicable whether or not a particular strain can be rendered stress resistant. As it is shown in present application L. johnsonii La1 - an already rather insensitive strain - could be rendered even more insensitive. This result was not predictable and thus can be considered to be unexpected. Thus, claims 1-3 meet the requirements of Art. 33(2)(3) PCT.
However, as regards claim 4 it is noted that at present no technical feature is apparent which would be suitable to render the claimed strain clearly and unambiguously novel over untreated ones (all facts and data presented in present application only concern Bifidobacteriae). Relating to this it is noted that the increased insensitivity towards stress only is a temporary property but not a permanent one. Therefore, at present novelty of claim 4 cannot be acknowledged (Art. 33(2)(3) PCT).

SECTION VI----------

Schmidt G. et al., International Journal of Food Microbiology, vol. 55 No. 1/3 pp. 41-45

Elkins J.G. et al. Applied and Environmental Microbiology, vol. 65, no. 10, October 1999, pp. 4594-4600

Lee S. et al., Current Microbiology, vol. 40, April 2000, pp. 283-287

SECTION VIII----------

The term "sublethal level" is relative and thus open to interpretation. Accordingly, the use of said expression renders the scope of claims containing it unclear.

In order to overcome this objection the subject-matter of claims 2 and/or 3 should be incorporated in claim 1.

Applicant: Société Des Produits Nestlé S.A.
Our file: 80295 WO

Claims

1. A method for protecting Lactobacillus johnsonii La1 against stress, which comprises the steps of treating said micro-organism with a sublethal level of stress selected from the group, which comprises thermal shock, osmotic shock, pH-shock, oxidative stress, chemical stress, nutritional stress, UV stress and cold stress.

2. The method of claim 1, which comprises the steps of treating with about 3,5 % NaCl for 15 minutes.

3. The method according to claim 1, which comprises the steps of treating at a temperature of about 48 °C for about 15 minutes.

4. A Lactobacillus johnsonni La1 obtained according to a method according to any of the preceding claims.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
NO 6592/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/05403	09/06/2000	11/06/1999
Applicant		
SOCIETE DES PRODUITS NESTLE S.A.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

BACTERIAL PROTECTION AGAINST STRESS

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[X] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

International Application No
PCT/EP 00/05403

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, EMBASE, MEDLINE, CAB Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. KILSTRUP ET AL.: "Induction of heat shock proteins DnaK, GroEL and GroES by salt stress in Lactococcus lactis." APPLIED AND ENVIROMENTAL MICROBIOLOGY, vol. 63, no. 5, May 1997 (1997-05), pages 1826-1837, XP002153669	1,2,4,5
Y	the whole document	3,6

X	FLAHAUT ET AL.: "Relationship between stress response towards bile salts, acid and heat treatment in Enterococcus faecalis." FEMS MICROBIOL. LETT, vol. 138, 1996, pages 49-54, XP000972031	1,2,4,5
Y	the whole document	3,6

	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 2000	15/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	U. VÖLKER ET AL.: "Stress protection and cross-protection by heat shock and salt stress in Bacillus subtilis." J. GEN. MICROBIOL., vol. 138, 1992, pages 2125-2135, XP000972204 the whole document	1,2,4,5
X	GANZLE M.G. ET AL: "Resistance of Escherichia coli and Salmonella against nisin and curvacin A." INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, (1 APR 1999) 48/1 (37-50). , XP000964926	1,4,5
Y	the whole document	2,3,6
X	ROCHA E.R. ET AL: "Characterization of a peroxide-resistant mutant of the anaerobic bacterium Bacteroides fragilis." JOURNAL OF BACTERIOLOGY, (1998) 180/22 (5906-5912). , XP000964951	1,4,5
Y	the whole document	2,3,6
X	M. GRESÍKOVÁ ET AL.: "Heat shock resistance in filial generations of marine Vibrio S14" BIOLOGICA, vol. 52, no. 6, 1997, pages 717-722, XP000964981 bratislava the whole document	1,4,5
X	DAVIS M.J. ET AL: "Acid tolerance in Listeria monocytogenes: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance." MICROBIOLOGY, (1996) 142/10 (2975-2982). , XP000964761 the whole document	1,4,5
X	SMITH, J. L. ET AL: "Relationship of water activity to prevention of heat injury in Staphylococcus aureus." LEBENSM.-WISS. U. TECHNOL.,, vol. 16, 1983, pages 195-197, XP000964927 the whole document	1,4,5
X	KRAMER G F ET AL: "Oxidative mechanisms of toxicity of low-intensity near-UV light in Salmonella typhimurium." JOURNAL OF BACTERIOLOGY, (1987 MAY) 169 (5) 2259-66. , XP000964770 the whole document	1,4,5

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHMIDT, G. ET AL: "Basic features of the stress response in three species of bifidobacteria: B. longum, B. adolescentis, and B. breve" INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, (2000) VOL. 55, NO. 1/3, PP. 41-45. 5 REF. MEETING INFO.: INTERNATIONAL SYMPOSIUM ON MICROBIAL STRESS AND RECOVERY I FOOD, QUIMPER, FRANCE, 14-16 JUNE, 1999. ISSN: 0168-1605, XP000964630 Nestle Research Center, Vers-chez-Les-Blanc, 1000 Lausanne 26, Switzerland. the whole document	1-6
P,X	J.G. ELKINS ET AL.: "Protective role of catalse in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 10, October 1999 (1999-10), pages 4594-4600, XP000964923 the whole document	1,4,5
P,X	S. LEE ET AL.: "HSP16.6 is involved in the development of thermotolerance and thylakoid stability in the unicellular cyanobacterium Synechocystis sp. PCC 6803" CURRENT MICROBIOLOGY, vol. 40, April 2000 (2000-04), pages 283-287, XP000964924 the whole document	1,4,5

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

STRAUS, Alexander
Becker, Kurig, Straus
Bavariastrasse 7
D-80336 Munich
SUISSE

Date of mailing (day/month/year)
17 août 2001 (17.08.01)

Applicant's or agent's file reference
NO 6592/WO

IMPORTANT NOTIFICATION

International application No.
PCT/EP00/05403

International filing date (day/month/year)
09 juin 2000 (09.06.00)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
SCHMIDT, Gudrun
Chemin de Bérée 56
CH- 1010 Lausanne
Switzerland

State of Nationality: DE
State of Residence: CH
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
SCHMIDT, Gudrun
Heidelbergerstr.10
D-70376 Stuttgart
Germany

State of Nationality: DE
State of Residence: DE
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Marie-José Devillard

Telephone No.: (41-22) 338.83.38

PA[illegible]T COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

STRAUS, Alexander
Becker, Kurig, Straus
Bavariastrasse 7
D-80336 Munich
SUISSE

Date of mailing (day/month/year)
23 July 2001 (23.07.01)

Applicant's or agent's file reference
NO 6592/WO

IMPORTANT NOTIFICATION

International application No.
PCT/EP00/05403

International filing date (day/month/year)
09 June 2000 (09.06.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address

LOCK, Graham
55, Avenue Nestlé
CH-1800 Vevey
Switzerland

State of Nationality

State of Residence

Telephone No.
+41 21 924 47 60

Facsimile No.
+41 21 924 28 80

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address

STRAUS, Alexander
Becker, Kurig, Straus
Bavariastrasse 7
D-80336 Munich
Switzerland

State of Nationality

State of Residence

Telephone No.
+49 89 746 303 0

Facsimile No.
+49 89 746 303 11

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

A. Karkachi

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

004167772

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
NO 6592/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 05403	09/06/2000	11/06/1999

Applicant

SOCIETE DES PRODUITS NESTLE S.A.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1 **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2 [ ] **Certain claims were found unsearchable** (See Box I).

3 [ ] **Unity of invention is lacking** (see Box II).

4 With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

BACTERIAL PROTECTION AGAINST STRESS

5 With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6 The figure of the **drawings** to be published with the abstract is Figure No. 1

[X] as suggested by the applicant.

[ ] None of the figures.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/[redacted] 00/05403

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, EMBASE, MEDLINE, CAB Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. KILSTRUP ET AL.: "Induction of heat shock proteins DnaK, GroEL and GroES by salt stress in Lactococcus lactis." APPLIED AND ENVIROMENTAL MICROBIOLOGY, vol. 63, no. 5, May 1997 (1997-05), pages 1826-1837, XP002153669	1,2,4,5
Y	the whole document	3,6

X	FLAHAUT ET AL.: "Relationship between stress response towards bile salts, acid and heat treatment in Enterococcus faecalis." FEMS MICROBIOL. LETT, vol. 138, 1996, pages 49-54, XP000972031	1,2,4,5
Y	the whole document	3,6

	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 2000	15/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	U. VÖLKER ET AL.: "Stress protection and cross-protection by heat shock and salt stress in Bacillus subtilis." J. GEN. MICROBIOL., vol. 138, 1992, pages 2125-2135, XP000972204 the whole document	1,2,4,5
X	GANZLE M.G. ET AL: "Resistance of Escherichia coli and Salmonella against nisin and curvacin A." INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, (1 APR 1999) 48/1 (37-50). , XP000964926	1,4,5
Y	the whole document	2,3,6
X	ROCHA E.R. ET AL: "Characterization of a peroxide-resistant mutant of the anaerobic bacterium Bacteroides fragilis." JOURNAL OF BACTERIOLOGY, (1998) 180/22 (5906-5912). , XP000964951	1,4,5
Y	the whole document	2,3,6
X	M. GRESÍKOVÁ ET AL.: "Heat shock resistance in filial generations of marine Vibrio S14" BIOLOGICA, vol. 52, no. 6, 1997, pages 717-722, XP000964981 bratislava the whole document	1,4,5
X	DAVIS M.J. ET AL: "Acid tolerance in Listeria monocytogenes: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance." MICROBIOLOGY, (1996) 142/10 (2975-2982). , XP000964761 the whole document	1,4,5
X	SMITH, J. L. ET AL: "Relationship of water activity to prevention of heat injury in Staphylococcus aureus." LEBENSM.-WISS. U. TECHNOL.,, vol. 16, 1983, pages 195-197, XP000964927 the whole document	1,4,5
X	KRAMER G F ET AL: "Oxidative mechanisms of toxicity of low-intensity near-UV light in Salmonella typhimurium." JOURNAL OF BACTERIOLOGY, (1987 MAY) 169 (5) 2259-66. , XP000964770 the whole document	1,4,5

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INTERNATIONAL SEARCH REPORT

Interna[redacted] Application No
PCT/[redacted] 00/05403

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHMIDT, G. ET AL: "Basic features of the stress response in three species of bifidobacteria: B. longum, B. adolescentis, and B. breve" INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, (2000) VOL. 55, NO. 1/3, PP. 41-45. 5 REF. MEETING INFO.: INTERNATIONAL SYMPOSIUM ON MICROBIAL STRESS AND RECOVERY I FOOD, QUIMPER, FRANCE, 14-16 JUNE, 1999. ISSN: 0168-1605, XP000964630 Nestle Research Center, Vers-chez-Les-Blanc, 1000 Lausanne 26, Switzerland. the whole document	1-6
P,X	J.G. ELKINS ET AL.: "Protective role of catalse in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 10, October 1999 (1999-10), pages 4594-4600, XP000964923 the whole document	1,4,5
P,X	S. LEE ET AL.: "HSP16.6 is involved in the development of thermotolerance and thylakoid stability in the unicellular cyanobacterium Synechocystis sp. PCC 6803" CURRENT MICROBIOLOGY, vol. 40, April 2000 (2000-04), pages 283-287, XP000964924 the whole document	1,4,5

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14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
80295 WO(AS/LS)		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/05403	09/06/2000	11/06/1999
International Patent Classification (IPC) or national classification and IPC C12N13/00		
Applicant SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30/12/2000	11.09.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer SCHEFFZYK, I Telephone No. +49 89 2399 8602

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/05403

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-12 as originally filed

Claims, No.:

1-4 as received on 15/06/2001 with letter of 15/06/2001

Drawings, sheets:

1/9-9/9 as originally filed

Sequence listing part of the description, pages:

1-3, filed with the letter of 120900

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-3
	No:	Claims	4
Inventive step (IS)	Yes:	Claims	4
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-4
	No:	Claims	

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

SECTION V----------

Claims 1-3 are deemed novel and inventive since firstly a method for protecting L. johnsonii La1 against stress is not taught in the available prior art and secondly, although it is known in the prior art that certain microorganisms can be rendered insensitive against stress it is not predicable whether or not a particular strain can be rendered stress resistant. As it is shown in present application L. johnsonii La1 - an already rather insensitive strain - could be rendered even more insensitive. This result was not predictable and thus can be considered to be unexpected. Thus, claims 1-3 meet the requirements of Art. 33(2)(3) PCT.
However, as regards claim 4 it is noted that at present no technical feature is apparent which would be suitable to render the claimed strain clearly and unambiguously novel over untreated ones (all facts and data presented in present application only concern Bifidobacteriae). Relating to this it is noted that the increased insensitivity towards stress only is a temporary property but not a permanent one. Therefore, at present novelty of claim 4 cannot be acknowledged (Art. 33(2)(3) PCT).

SECTION VI----------

Schmidt G. et al., International Journal of Food Microbiology, vol. 55 No. 1/3 pp. 41-45

Elkins J.G. et al. Applied and Environmental Microbiology, vol. 65, no. 10, October 1999, pp. 4594-4600

Lee S. et al., Current Microbiology, vol. 40, April 2000, pp. 283-287

SECTION VIII----------

The term "sublethal level" is relative and thus open to interpretation. Accordingly, the use of said expression renders the scope of claims containing it unclear.

In order to overcome this objection the subject-matter of claims 2 and/or 3 should be incorporated in claim 1.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 December 2000 (21.12.2000)

PCT

(10) International Publication Number
WO 00/77186 A3

(51) International Patent Classification[7]: C12N 1/20, 1/04

(21) International Application Number: PCT/EP00/05403

(22) International Filing Date: 9 June 2000 (09.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/138,946 11 June 1999 (11.06.1999) US

(71) Applicant *(for all designated States except US)*: SOCIETE DES PRODUITS NESTLE S.A. [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SCHMIDT, Gudrun [DE/CH]; Chemin de Bérée 56, CH- 1010 Lausanne (CH). ZINK, Ralf [DE/CH]; Chemin de la Maison Jean 36, CH-1801 Le Mont Pélerin (CH).

(74) Agent: LOCK, Graham; 55, Avenue Nestlé, CH-1800 Vevey (CH).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

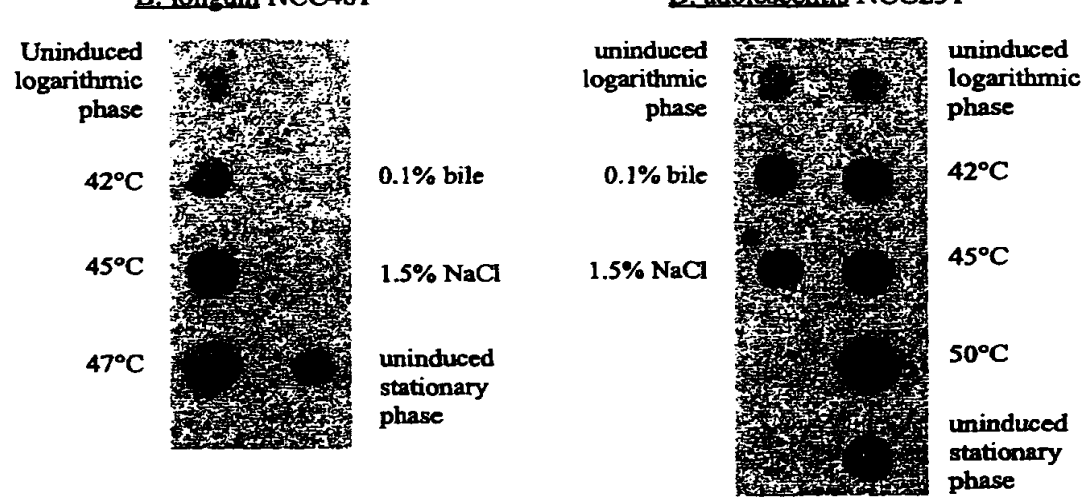
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
28 June 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: BACTERIAL PROTECTION AGAINST STRESS



(57) Abstract: A bacterial cell having protection against conditions which are lethal to an unprotected bacterial cell wherein, the protected cell is obtained by subjecting a bacterial cell to treatment with a sublethal level of stress.

WO 00/77186 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/05403

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, EMBASE, MEDLINE, CAB Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. KILSTRUP ET AL.: "Induction of heat shock proteins DnaK, GroEL and GroES by salt stress in Lactococcus lactis." APPLIED AND ENVIROMENTAL MICROBIOLOGY, vol. 63, no. 5, May 1997 (1997-05), pages 1826-1837, XP002153669	1,2,4,5
Y	the whole document	3,6

X	FLAHAUT ET AL.: "Relationship between stress response towards bile salts, acid and heat treatment in Enterococcus faecalis." FEMS MICROBIOL. LETT, vol. 138, 1996, pages 49-54, XP000972031	1,2,4,5
Y	the whole document	3,6

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 2000	15/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/05403

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	U. VÖLKER ET AL.: "Stress protection and cross-protection by heat shock and salt stress in Bacillus subtilis." J. GEN. MICROBIOL., vol. 138, 1992, pages 2125-2135, XP000972204 the whole document	1,2,4,5
X	GANZLE M.G. ET AL: "Resistance of Escherichia coli and Salmonella against nisin and curvacin A." INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, (1 APR 1999) 48/1 (37-50). , XP000964926	1,4,5
Y	the whole document	2,3,6
X	ROCHA E.R. ET AL: "Characterization of a peroxide-resistant mutant of the anaerobic bacterium Bacteroides fragilis." JOURNAL OF BACTERIOLOGY, (1998) 180/22 (5906-5912). , XP000964951	1,4,5
Y	the whole document	2,3,6
X	M. GRESÍKOVÁ ET AL.: "Heat shock resistance in filial generations of marine Vibrio S14" BIOLOGICA, vol. 52, no. 6, 1997, pages 717-722, XP000964981 bratislava the whole document	1,4,5
X	DAVIS M.J. ET AL: "Acid tolerance in Listeria monocytogenes: The adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance." MICROBIOLOGY, (1996) 142/10 (2975-2982). , XP000964761 the whole document	1,4,5
X	SMITH, J. L. ET AL: "Relationship of water activity to prevention of heat injury in Staphylococcus aureus." LEBENSM.-WISS. U. TECHNOL.,, vol. 16, 1983, pages 195-197, XP000964927 the whole document	1,4,5
X	KRAMER G F ET AL: "Oxidative mechanisms of toxicity of low-intensity near-UV light in Salmonella typhimurium." JOURNAL OF BACTERIOLOGY, (1987 MAY) 169 (5) 2259-66. , XP000964770 the whole document	1,4,5

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHMIDT, G. ET AL: "Basic features of the stress response in three species of bifidobacteria: B. longum, B. adolescentis, and B. breve" INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, (2000) VOL. 55, NO. 1/3, PP. 41-45. 5 REF. MEETING INFO.: INTERNATIONAL SYMPOSIUM ON MICROBIAL STRESS AND RECOVERY I FOOD, QUIMPER, FRANCE, 14-16 JUNE, 1999. ISSN: 0168-1605, XP000964630 Nestle Research Center, Vers-chez-Les-Blanc, 1000 Lausanne 26, Switzerland. the whole document	1-6
P,X	J.G. ELKINS ET AL.: "Protective role of catalse in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 10, October 1999 (1999-10), pages 4594-4600, XP000964923 the whole document	1,4,5
P,X	S. LEE ET AL.: "HSP16.6 is involved in the development of thermotolerance and thylakoid stability in the unicellular cyanobacterium Synechocystis sp. PCC 6803" CURRENT MICROBIOLOGY, vol. 40, April 2000 (2000-04), pages 283-287, XP000964924 the whole document	1,4,5

REPLACED BY
ART 34 AMDT

Claims

1. A bacterial cell having protection against conditions which are lethal to an unprotected bacterial cell wherein, the protected cell is obtained by subjecting a bacterial cell to treatment with a sublethal level of stress.

2. A bacterial cell according to claim 1 selected from the group which comprises bifidobacteria, lactic acid bacteria, enterococci, streptomyces and bacilli.

3. A bacterial cell according to claim or 2 selected from the group which comprise Bifidobacterium longum NCC481, Bifidobacterium adolescentis NCC251 and Lactobacillus johnsonii La1.

4. A nutritive composition which comprises bacteria having protection against conditions which are lethal to unprotected bacteria wherein, the protected bacteria are obtained by subjecting bacteria to treatment with a sublethal level of stress.

5. A method of protecting a bacterial cell against stress which comprises the steps of treating a bacterial cell with a sublethal level of stress selected from the group which comprises thermal shock, osmotic shock, pH shock, oxidative stress, chemical stress nutritional stress, UV-stress, and cold stress.

6. A method according to claim 5 which comprises the step of treating with about 0.01 to about 0.1% salt for about 30 minutes.

Bacterial Protection

The present invention relates to a bacterial cell having protection against stress including the affects of extreme temperature change and osmotic shock; a nutritive or medicinal composition comprising the protected bacterial cell; and a method of protecting bacteria against stress.

Within the context of this specification the word "comprises" is taken to mean "includes, among other things". It is not intended to be construed as "consists of only". In addition, the word "stress" is used interchangeably with the term "adverse conditions". It includes, but is not limited to, adverse conditions of temperature (heat shock, cold shock), salt (osmotic shock), pH (pH shock), chemical stresses (antibiotics, alcohol, H2O2, etc.), nutritional stress, UV-stress, cold stress and oxygen concentration (oxidative stress).

Standard amino acid, RNA and DNA codes are used within this specification which are defined by the IUB Biochemical Nomenclature Commission.

It is well known that bacteria such as lactic acid bacteria (LAB) are ubiquitously found in the environment and they are largely used for the production of fermented products. For example, in the food industry bacteria are used in fermentation of milk products and production of starter cultures. During production of starter cultures, food fermentation, manufacture and storage, the bacteria that are employed must deal with different kinds of adverse conditions which generally have the effect of dramatically reducing their viability, stability and activity. These adverse conditions vary with production requirements and include thermal shock (freeze-drying or spray-drying), osmotic shock (drying) and pH shock (fermentation). It will be appreciated that the susceptibility or inability of bacteria to cope with these stresses is a problem in cases where bacteria are used on a large scale.

The presence of Bifidobacteria or lactobacilli in the human intestine, primarily the small and large intestine, is generally accepted as a contributing factor for a healthy well-being. In addition, it is considered that Bifidobacteria and lactobacilli may be useful in prophylaxis or treatment of ailments including

gastrointestinal infections. In the light of this, it has been suggested that large populations of Bifidobacteria and lactobacilli in the intestine should be maintained and products comprising the bacteria should be administered. Often these products comprise different species of Bifidobacteria or lactobacilli. However, the stresses that Bifidobacteria and lactobacilli are exposed to during manufacture and storage of the products can significantly reduce their viability and/or physiological activity.

The natural response by bacterial cultures to sublethal temperature shifts or other sublethal stresses (including exposure to oxygen and osmotic shock) includes rapid expression of a distinct set of polypeptides called "stress-proteins". These proteins have been shown to enable Gram-positive bacteria such as for example *Lactococcus lactis*, *Bacillus subtilis*, *Lactobacillus acidophilus*, *Lactobacillus sakei*, *Enterococcus faecalis*, and *Lactobacillus johnsonii* to adapt to otherwise growth-limiting conditions.

One of the most studied stress proteins are the heat shock proteins or chaperones. These proteins are generally involved in the maturation of newly synthesised proteins, and they assist in refolding of denatured proteins. Numerous stress-response genes have been characterised in LAB, including those encoding the two major chaperone machines (groES/groEL and hrcA/grpE/dnaK/dnaJ) involved in the proper folding of newly synthesised proteins and the repair of those that are denatured.

Remarkably, it has now been found that bacteria, including Bifidobacteria and lactobacilli, can be protected against levels of stress that are lethal in unprotected bacteria. Surprisingly, this can be done by subjecting the bacteria to a sublethal level of stress treatment. It has surprisingly been found that after this initial stress treatment a higher level of stress is required to adversely affect the bacteria. This is unexpected because it was thought that cells which are damaged by stress would be less likely to cope with additional stress. In fact, the converse has been found – pre-stressed cells are able to bear a higher stress level compared to control cells which have not been pre-stressed.

Protection against one form of stress acquired by treatment with a dissimilar form of stress has been referred to as "cross-protection". This is unexpected

because it was thought that cells damaged by treatment with one stress should render them more sensitive against an additional sublethal or lethal stress.

Accordingly, in a first aspect the invention provides a bacterial cell having protection against conditions which are lethal to an unprotected bacterial cell wherein, the protected cell is obtained by subjecting a bacterial cell to treatment with a sublethal level of stress.

In a second aspect, the invention provides a nutritive composition which comprises bacteria having protection against conditions which are lethal to unprotected bacteria wherein, the protected bacteria are obtained by subjecting bacteria to treatment with a sublethal level of stress and allowing them to recover.

In a further aspect, the invention provides a method of protecting a bacterial cell against stress which comprises the steps of treating a bacterial cell with a sublethal level of stress selected from the group which comprises thermal shock, osmotic shock, pH shock, oxidative stress, chemical stress, nutritional stress, UV-stress, cold stress.

Preferably the method includes the additional step of allowing the cell to recover.

Preferably chemical stress is provided by treatment with antibiotics, alcohol or H_2O_2.

Preferably, the bacterial cell is selected from the group which comprises Bifidobacteria, lactic acid bacteria, enterococci, streptomyces, and bacilli.

More preferably, the bacterial cell is *Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium breve* or *Lactobacillus johnsonii*. An advantage provided by these bacteria is that they have the ability to rapidly acidify their substrate, therefore producing microbiologically safe products. In addition they contribute to a healthy well-being in humans and animals. Furthermore, they display a protective role against attack by enteric pathogens and are associated with anti-carcinogenic, anti-mutagenic and anti-tumorgenic activities. Without wishing to be bound by theory, recent reports suggest that they might act directly

in the intestinal tract through antimicrobial activity, indirectly through immunomodulation via intestinal cells or by modifying the function of the normal indigenous microflora.

Preferably bacteria, more preferably Bifidobacteria and lactobacilli, are treated with sublethal salt concentrations to protect them against otherwise lethal salt concentrations or the cells are treated with sublethal thermal stress to protect them against otherwise lethal temperatures. Furthermore, results show that treatment with salt (e.g. NaCl) protect these bacteria against lethal thermal stress or against lethal cycles of freeze-thawing. Accordingly, the invention alternatively includes the steps of treating cells with salt to protect against thermal stress or treating the cells with adverse temperature conditions to protect against salt stress.

Preferably the bacterial cells are selected from *Bifidobacterium longum*, *Bifidobacterium adolescentis* or *Lactobacillus johnsonii*. More preferably the bacterial cells are selected from *Bifidobacterium longum* NCC481, *Bifidobacterium adolescentis* NCC251 or *Lactobacillus johnsonii* La1.

Preferably, protection against lethal salt concentrations (eg of between 0.1% and 0.4%) is carried out by treatment with about 0.01 to about 0.1% salt for about 15 to about 60 min. Preferably the salt is bile salt.

Preferably protection against lethal thermal stress (eg of between about 50°C to about 60°C) is carried out by treatment at about 37°C to about 50°C for about 15 to about 60 min or by treatment with a salt concentration of between about 1% and about 4% for about 30 to about 60 min.

Preferably, protection against freeze-thawing (eg 1 to 10 cycles) is carried out by treatment of the cells with salt concentration of between 1% and 4%.

Preferably *Bifidobacterium longum* NCC481 cells are protected. More preferably, protection of *Bifidobacterium longum* NCC481 cells is carried out in the logarithmic phase of their growth cycle against lethal bile salt concentrations (eg of between about 0.2% and about 0.3% for 30 min) by subjecting the cells to about 0.1% bile salt for about 30 min before lethal challenge.

Preferably, protection of *Bifidobacterium longum* NCC481 cells is carried out in the stationary phase of their growth cycle against lethal bile salt concentrations (eg of about 0.075% and about 0.15% for about 30 min) by treatment of the cells with about 0.05% bile salt for about 30 min before lethal challenge.

Preferably, *Bifidobacterium adolescentis* NCC251 cells are protected. More preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the logarithmic phase of their growth cycle against lethal bile salt concentrations (eg of between about 0.3% and about 0.4% for about 30 min) by subjecting the cells to about 0.1% bile salt for about 30 min before lethal challenge.

Preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the stationary phase of their growth cycle against a lethal bile salt concentration (eg of about 0.15% for about 30 min) by subjecting the cells to about 0.1% bile salt for about 30 min before lethal challenge

Preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the stationary phase of their growth cycle against the otherwise lethal effect of (eg about 3 to about 4 cycles) freeze-thawing (about -80°C to about room temperature (preferably about 20°C to about 30°C, more preferably 25°C)) by subjecting the cells to about 2% of NaCl for about 1 h.

Preferably, protection of *Bifidobacterium adolescentis* NCC251 cells is carried out in the logarithmic phase of their growth cycle against an otherwise lethal temperature of 55°C for 20 min by treatment of the cells for about 30 min at about 45°C, about 15 min at about 47°C or for about 1 h with 1% or 2% NaCl.

Preferably *Lactobacillus johnsonii* La1 cells are protected. More preferably, protection of *Lactobacillus johnsonii* La1 cells is carried out in the logarithmic phase of their growth cycle against an otherwise lethal temperature of 55°C for up to 1h by treatment of the cells with about 3.5% NaCl for about 15 min or about 48°C for about 15 min.

Preferably, protection of *Lactobacillus johnsonii* La1 cells is carried out in the stationary phase of their growth cycle against an otherwise lethal temperature of 55°C for up to 1h by treatment of the cells with a temperature of about 48°C for about 15 min or with about 3.5% NaCl for about 15 min.

Embodiments of the invention will now be described in further detail with reference to the accompanying drawings in which:

Figure 1 shows results of a dot blot hybridization of RNA from cells of *Bifidobacterium longum* NCC481 and *Bifidobacterium adolescentis* NCC251 after 10 min exposure to different kinds of stress. Hybridization was performed using the specific probes GSR8 and GSR5 for NCC481 and NCC251, respectively.

Figure 2 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 at 55°C after different pre-inductions in the logarithmic phase. Cells were grown in MRS and cysteine at 37°C to an OD600 of between 0.4 and 0.7. Aliquots were taken and subjected for 15 min to 47°C, for 30 min to 45°C, or 1 h to 1.5% NaCl or 2% NaCl; the control remained at 37°C. The samples were shifted to 55°C and after 10 and 20 min the viable cell counts were determined.

Figure 3 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 after three and four cycles of freeze-thawing. Stationary phase cells were taken and subjected for 1 h to 2% NaCl, the control remained without salt addition. The samples were shifted to -80°C and thawed at room temperature. This cycle was repeated three and four times before the viable cell counts were determined.

Figure 4 shows a graph of survival of *Bifidobacterium longum* NCC481 under lethal bile salt conditions in the logarithmic phase. Cells were grown to an OD600 (optical density at 600nm) between 0.4 and 0.7 and subjected for 30 min to 0.1% Oxgall. The control remained without Oxgall addition. The samples were aliquoted and shifted to 0.2%, 0.25%, and 0.3% Oxgall for 30 min, and the viable cell counts were determined.

Figure 5 shows a graph of survival of *Bifidobacterium longum* NCC481 under lethal bile salt conditions in the stationary phase. Cells were subjected for 30 min to an 0.05% Oxgall-treatment. The control remained without any Oxgall addition. The samples were aliquoted and shifted to 0.075%, 0.1%, and 0.15% Oxgall for 30 min, and the viable cell counts were determined.

Figure 6 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 under lethal bile salt conditions in the logarithmic phase. Cells were grown to an OD600 (optical density at 600nm) between 0.4 and 0.7 and subjected for 30 min to an 0.1% Oxgall-treatment. The control remained without any Oxgall addition. The samples were aliquoted and shifted to 0.3% and 0.4% Oxgall for 30 min, and the viable cell counts were determined.

Figure 7 shows a graph of survival of *Bifidobacterium adolescentis* NCC251 under lethal bile salt conditions in the stationary phase. Cells were subjected for 30 min to an 0.1% Oxgall-treatment. The control remained without any Oxgall addition. The samples were aliquoted and shifted to 0.15% Oxgall for 30 min, and the viable cell counts were determined.

Figure 8 shows a graph of survival of *Lactobacillus johnsonii* La1under lethal thermal conditions. Cells were grown in MRS at 37°C to an OD600 (optical density at 600nm) between 0.4 and 0.7. Samples were taken and subjected to 3.5% NaCl or 48°C for 15 min. The control remained at 37°C. Afterwards the samples were shifted to 55°C and the viable cell counts were determined after 30 min and 60 min.

Figure 9 shows a graph of survival of *Lactobacillus johnsonii* La1 in the stationary phase of their growth cycle under lethal thermal conditions. Samples were taken and subjected to 3.5% NaCl or 48°C for 15 min. The control remained at 37°C. Afterwards the samples were shifted to 55°C and the viable cell counts were determined after 60 min.

Strains and growth conditions

Bifidobacterium adolescentis NCC251, *Bifidobacterium longum* NCC481, *Bifidobacterium longum* NCC490, *Bifidobacterium longum* NCC585, and

Bifidobacterium breve NCC298 were cultivated in MRS medium supplemented with 0.5 g/l cysteine at 37°C under anaerobic conditions (98% nitrogen and 2% hydrogen). *Lactococcus lactis* MG1363 was grown in MRS medium at 30°C. *Escherichia coli* TG1 (Amersham) was cultivated in Luria-Bertani medium at 37°C. *Lactobacillus johnsonii* La1 was grown in MRS at 37°C.

Stress treatment

Cells were grown to an OD600 (optical density at 600nm) between 0.4 and 0.7 or taken in the stationary phase and subjected for different times to various stress conditions. Cells used for freeze-thawing experiments were concentrated in saline solution before being subjected to –80°C. Salt stress was exerted by adding sodium chloride to the samples while for bile-salt stress OXGALL (Trade Mark) (Difco) was used.

The stress treatment of Bifidobacteria was performed under anaerobic conditions while the determination of viable cells was carried out under aerobic conditions. Cells of lactobacilli were grown under microaerophil conditions, stress treatments and determination of viable cell counts was performed under aerobic conditions.

Bifidobacteria

Ranges for inductions and lethal challenges

	re-induction	lethal challenge
pH (e.g. HCl):	pH 6.0-3.5	pH 2.5-2
Bile (e.g. Oxgall):	0.01%-0.1%	0.075%-0.4%
Temperature:	37°C-48°C	50°C-60°C
Salt (e.g. NaCl):	0.5%-3%	3%-8%

Time of pre-induction and lethal challenge can vary dependent on strain and stress conditions between 5 min to 2 h.

Lactobacilli

Ranges for inductions and lethal challenges

	re-induction	lethal challenge
pH (e.g. HCl, lactic acid)	pH 6.0-4.5	pH 4.0-2
Temperature	40°C-50°C	50°C-60°
Salt (e.g. NaCl)	0.5%-3.5%	4%-8%

Time of pre-induction and lethal challenge can vary dependent on strain and stress conditions between 5 min to 2 h

DNA techniques

Isolation of chromosomal DNAs was carried out according to standard methods.

Analyses of mRNA

For Dot-blot hybridisation total RNA was isolated, denatured and transferred to uncharged nylon membranes (GeneScreen, NEN) according to standard methods. The membranes were pre-hybridised (1h, 40°C) and subsequently hybridised for 4h with 100 pmol DIG-labelled probes (Boehringer). The membranes were washed twice for 5 min in 2x SSC containing 0.1% SDS at 40°C and once at the probe-dependent temperature, which was 46°C and 48°C, respectively for the two dnaK specific probes GSR5 (5′-CATCGAAGGTGCCGCCAC-3′) and GSR8 (5′-TCGTCACCACCGAGGTG-3′), and 51°C for the universal probe 1028R (5′-CCTTCTCCCGAAGTTACGG-3′). Detection was performed according to the manufacturers instructions.

PCR amplification

The core dnaK region was amplified using the degenerate primers HS1 (5′-ATIACIGTICCIGCITA (T/C)TT(T/C)AA(T/C)GA-3′) and HS2 (5′-CATIGT(T/C)TCIATICCIA(A/G)IGAIA(G/A)IGG-3′) as well as 1 µg of chromosomal DNA as template. Amplification reactions were performed in a total volume of 100 µl (containing 200 µM each of dATP, dCTP, dGTP, and dTTP, 50 pmol of each primer, 2.5 U of Super-Taq DNA Polymerase (HT Biotechnology), and the corresponding 1x PCR buffer). Reactions were carried out with a Perkin-Elmer thermocycler: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

Identification of the dnaK gene

Based on the alignment of Barril et al. (1994), we chose two regions of the DnaK of *Lactococcus lactis, Escherichia coli* and *Bacillus megaterium* possessing identical amino acid sequences and designed two degenerate primers HS1 and HS2 corresponding to the amino acids at positions 114 to 122 and 366 to 374 of *Lactococcus lactis* DnaK, respectively. This primer pair was used for a PCR-amplification using chromosomal DNA of NCC481, NCC490, NCC585, NCC251, and NCC298 as templates. Two fragments were obtained for each strain. For all strains those fragments corresponding in size to that of the two positive controls *Escherichia coli* and *Lactococcus lactis* were isolated from an agarose gel, purified and sequenced. In each fragment an open reading frame was identified showing high sequence similarities to the core region of known DnaK proteins. Particularly high identities were observed to streptomyces and mycobacteria as well as to *Lactobacillus sakei*, bacilli, and streptococci.

mRNA analysis of *dnaK* gene expression

The transcriptional induction of dnaK was investigated with cells exposed to heat shock and to additional general stress conditions. *Bifidobacterium longum* NCC481 and *Bifidobacterium adolescentis* NCC251 cells of the logarithmic phase were subjected to 0.1% bile salt, 1.5% NaCl or a heat shock for 10 min at 42°C and 45°C. Maximum temperatures of 47°C and 50°C were tested for NCC481 and NCC251, respectively. Uninduced cells from the logarithmic and stationary phase were always used as controls. Total RNA was isolated and subjected to dot blot hybridization. The dnaK specific probes GSR8 and GSR5 were used for NCC481 and NCC251, respectively. The universal probe 1028R was chosen to verify the amount and quality of RNA on the membrane. An increased concentration of dnaK specific mRNA was observed when subjecting the cells to increasing temperatures (Figure 1). In contrast to NCC251, dnaK of NCC481 was only slightly induced in cells entering the stationary phase. Furthermore a slight induction of dnaK was observed in NCC251 after bile-salt and NaCl treatment. No significant induction under identical conditions was obtained for NCC481.

Survival and cross-protection

Growth and survival of *Lactobacillus johnsonii* La1, *Bifidobacterium adolescentis* NCC251 and *Bifidobacterium longum* NCC481 at different temperature, bile-salts and salt conditions were tested.

Remarkably, logarithmic phase NCC251 showed an increased resistance to the generally lethal temperature of 55°C after being treated with sublethal heat stress. An almost 24-fold and 128–fold higher thermotolerance was observed after subjecting the cells to 47°C for 15 min prior to a heat shock for 10 min and 20 min, respectively (Figure 2). These figures are remarkable because they show how that, unexpectedly effective pre-induction of cells can be to protect them against otherwise lethal challenges. A 9-fold and 15-fold cross-protection of cells against 55°C was achieved by pretreatment for 1h with 1.5% NaCl. An equal protection against thermal stress could also be observed by pre-inducing at 45°C for 30 min or 2% NaCl for 1 h (Figure 2).

Cells in the logarithmic phase of the growth cycle of *Lactobacillus johnsonii* La1 showed a 400-fold higher protection against 55°C for 30 min after being pretreated with 3.5% NaCl or 15 min 48°C. After one hour at 55°C, a 10-fold and 5-fold higher protection was observed against 55°C in samples pretreated with 3.5% NaCl and 48°C for 15 min, respectively (Figure 8).

Stationary phase cells of *Lactobacillus johnsonii* La1 showed a remarkable 20-fold higher protection against 55°C for 1 hour after being treated with 3.5% NaCl for 15 minutes at 48°C (Figure 9).

Cells of *Bifidobacterium adolescentis* NCC251 in the stationary phase demonstrated a 14-fold higher survival after continuous cycles of freeze-thawing if pre-stressed with 2% NaCl for 1h (Figure 3). After 4 cycles of freeze thawing a 10-fold higher survival was observed.

Protection against lethal bile-salt concentrations could be observed in the logarithmic as well as in the stationary phase of *Bifidobacterium adolescentis* NCC251 and Bifidobacterium longum NCC481. A preconditioning (e.g. 30 min) of logarithmic cells with 0.1% bile-salts resulted in a 300-fold and 21-fold

protection against 0.3% and 0.4% bile-salts in logarithmic phase cells of *Bifidobacterium adolescentis* NCC251, respectively, (Figure 6). An 81-fold increased survival of stationary phase cells of *Bifidobacterium adolescentis* NCC251 (pre-induced with 0.1% bile-salts) was observed under the lethal concentration of 0.15% bile-salts (Figure 7). Analogous results were obtained for *Bifidobacterium longum* NCC481. Logarithmic cells, pre-induced with 0.1% Oxgall showed a 400-fold, 1800-fold, and 580-fold better survival against the lethal concentrations of 0.2%, 0.25%, and 0.3% Oxgall, respectively (Figure 4).Cells of the stationary phase showed a 3-fold, 29-fold, and 150-fold better survival for 30 min against 0.075%, 0.1%, and 0.15% Oxgall when they were pre-induced for 30 min with 0.05% Oxgall (Figure 5).

In contrast to the results published by Flahaut *et al.* (1996) where a protection of *Enterococcus faecalis* cells against 0.3% bile salts could only be achieved for 30 seconds, remarkably cells were able to be protected for 30 min against lethal bile salt concentration. This could not have been predicted.

The core region of dnaK of *Bifidobacterium longum* NCC481, *Bifidobacterium longum* NCC490, *Bifidobacterium longum* NCC585, *Bifidobacterium adolescentis* NCC251, and *Bifidobacterium breve* NCC298 were PCR-amplified and identified. Subsequent mRNA analyses revealed that in NCC251 and NCC481 the induction of dnaK is regulated at the transcriptional level. Transcription is generally induced by heat and for NCC251 also by treatment with salt and bile-salts.

In the light of these findings it has been concluded that stress pre-treatment of Bifidobacteria and/or lactobacilli can lead to a significantly increased chances of survival under otherwise lethal homologous or heterologous stress conditions.

Claims

1. A bacterial cell having protection against conditions which are lethal to an unprotected bacterial cell wherein, the protected cell is obtained by subjecting a bacterial cell to treatment with a sublethal level of stress.

2. A bacterial cell according to claim 1 selected from the group which comprises bifidobacteria, lactic acid bacteria, enterococci, streptomyces and bacilli.

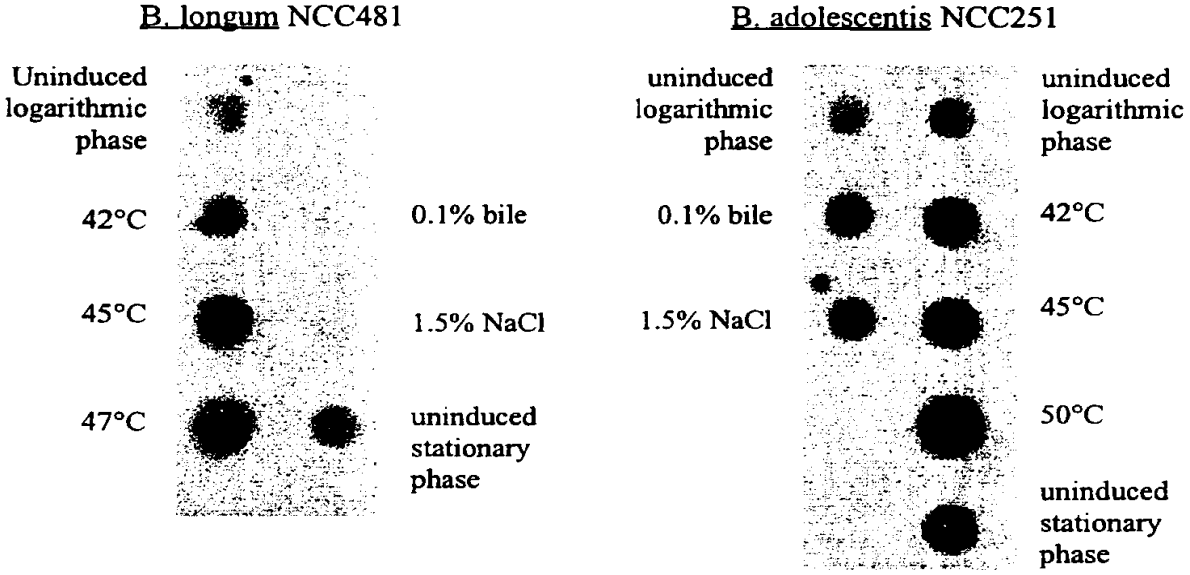
3. A bacterial cell according to claim or 2 selected from the group which comprise Bifidobacterium longum NCC481, Bifidobacterium adolescentis NCC251 and Lactobacillus johnsonii La1.

4. A nutritive composition which comprises bacteria having protection against conditions which are lethal to unprotected bacteria wherein, the protected bacteria are obtained by subjecting bacteria to treatment with a sublethal level of stress.

5. A method of protecting a bacterial cell against stress which comprises the steps of treating a bacterial cell with a sublethal level of stress selected from the group which comprises thermal shock, osmotic shock, pH shock, oxidative stress, chemical stress nutritional stress, UV-stress, and cold stress.

6. A method according to claim 5 which comprises the step of treating with about 0.01 to about 0.1% salt for about 30 minutes.

Figure 1


B. longum NCC481
Uninduced logarithmic phase
42°C
45°C
47°C
0.1% bile
1.5% NaCl
uninduced stationary phase
B. adolescentis NCC251
uninduced logarithmic phase
0.1% bile
1.5% NaCl
uninduced logarithmic phase
42°C
45°C
50°C
uninduced stationary phase

5

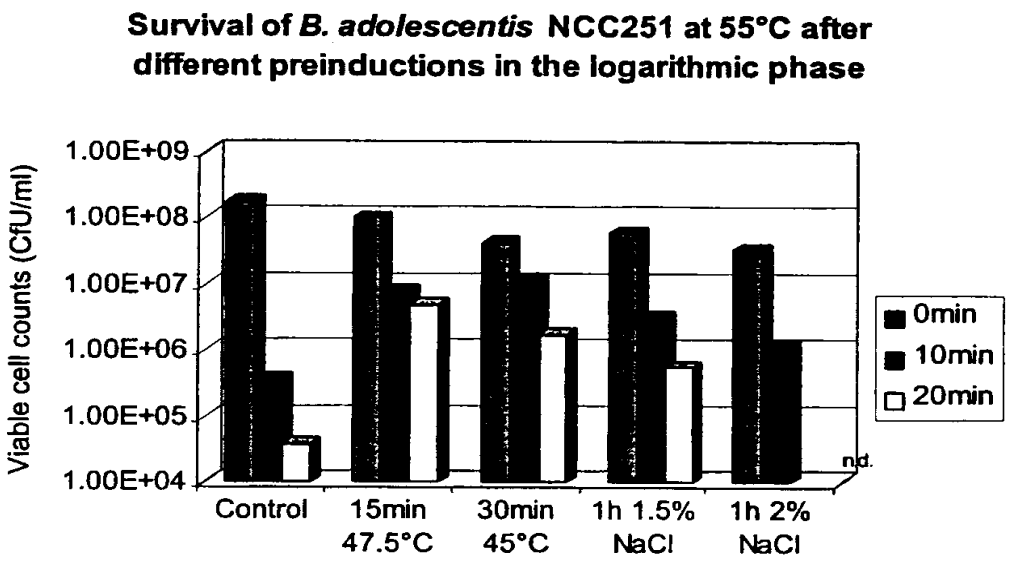
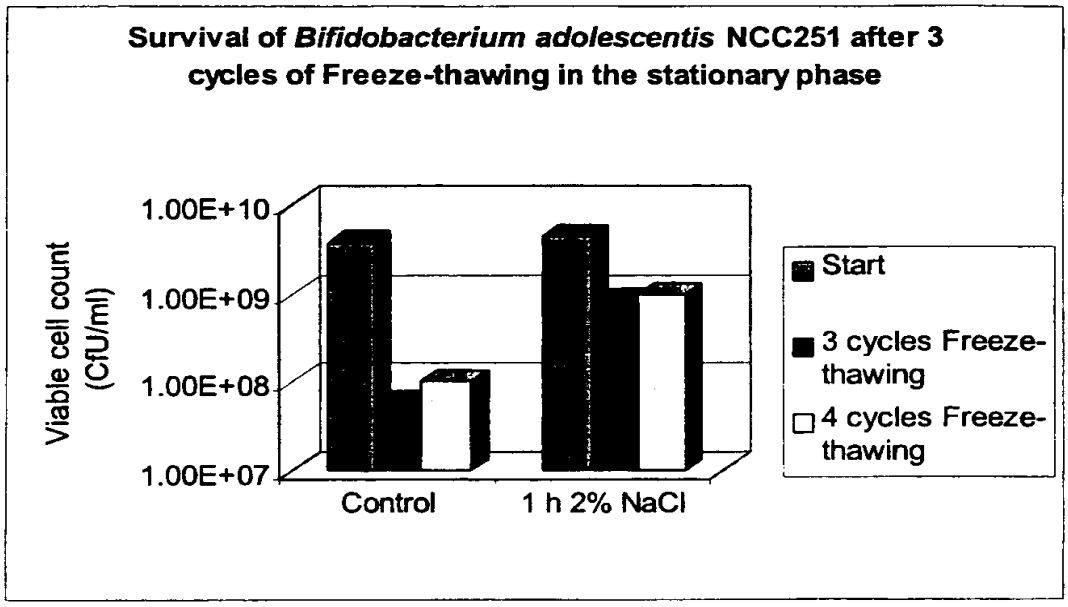

Survival of B. adolescentis NCC251 at 55°C after different preinductions in the logarithmic phase
Viable cell counts (CfU/ml)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
n.d.
Control
15min 47.5°C
30min 45°C
1h 1.5% NaCl
1h 2% NaCl
0min
10min
20min

Figure 2

Figure 3


Survival of *Bifidobacterium adolescentis* NCC251 after 3 cycles of Freeze-thawing in the stationary phase
Viable cell count (CfU/ml)
1.00E+10
1.00E+09
1.00E+08
1.00E+07
Control
1 h 2% NaCl
Start
3 cycles Freeze-thawing
4 cycles Freeze-thawing

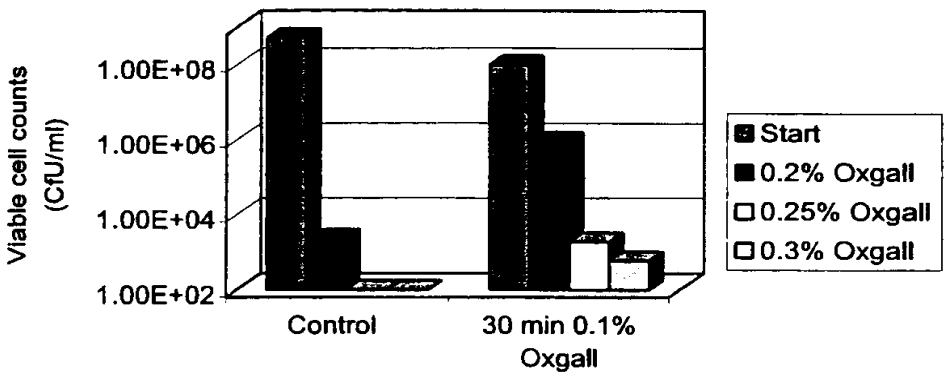

Viable cell counts (CfU/ml)
1.00E+08
1.00E+06
1.00E+04
1.00E+02
Control
30 min 0.1% Oxgall
Start
0.2% Oxgall
0.25% Oxgall
0.3% Oxgall

Figure 4

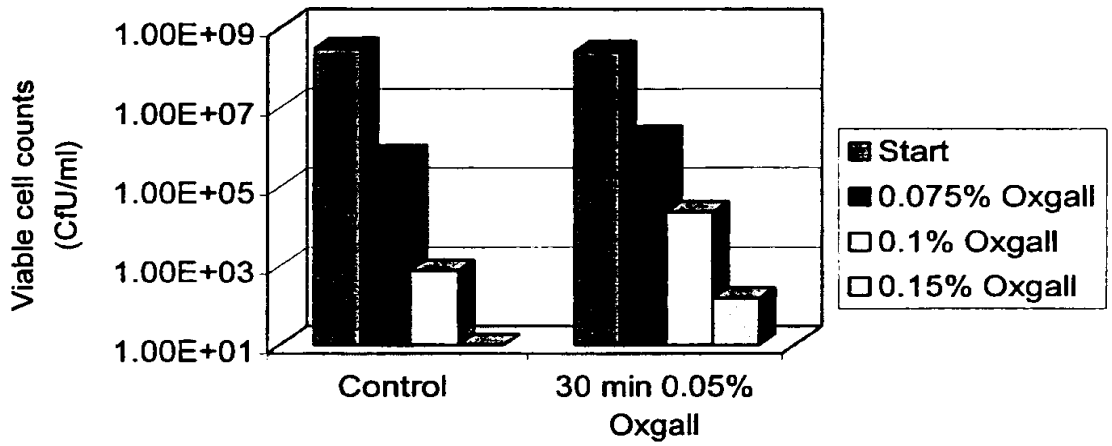

Viable cell counts (CfU/ml)
1.00E+09
1.00E+07
1.00E+05
1.00E+03
1.00E+01
Control
30 min 0.05% Oxgall
Start
0.075% Oxgall
0.1% Oxgall
0.15% Oxgall

Figure 5

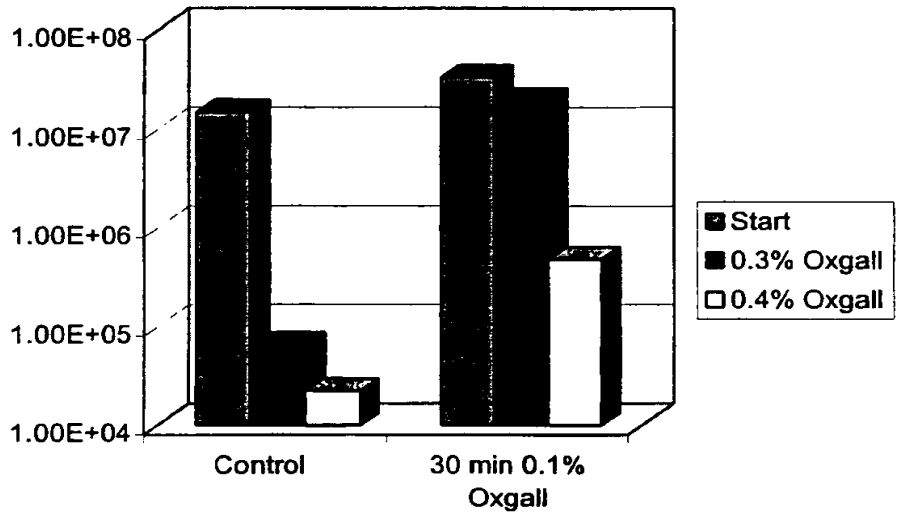

1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Control
30 min 0.1% Oxgall
Start
0.3% Oxgall
0.4% Oxgall

Figure 6

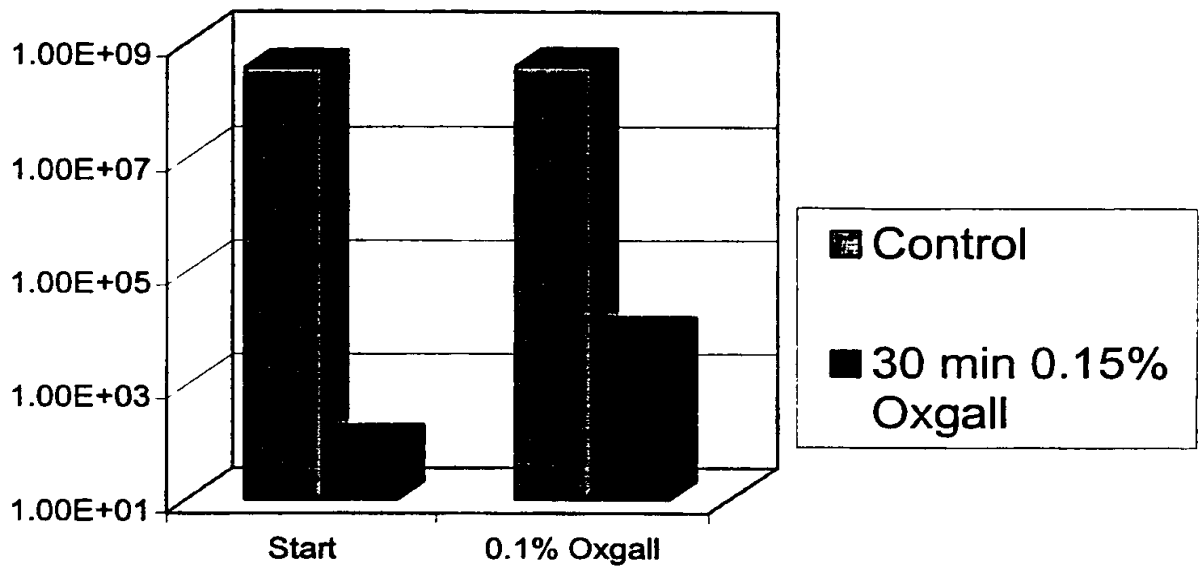

1.00E+09
1.00E+07
1.00E+05
1.00E+03
1.00E+01
Start
0.1% Oxgall
Control
30 min 0.15% Oxgall

Figure 7

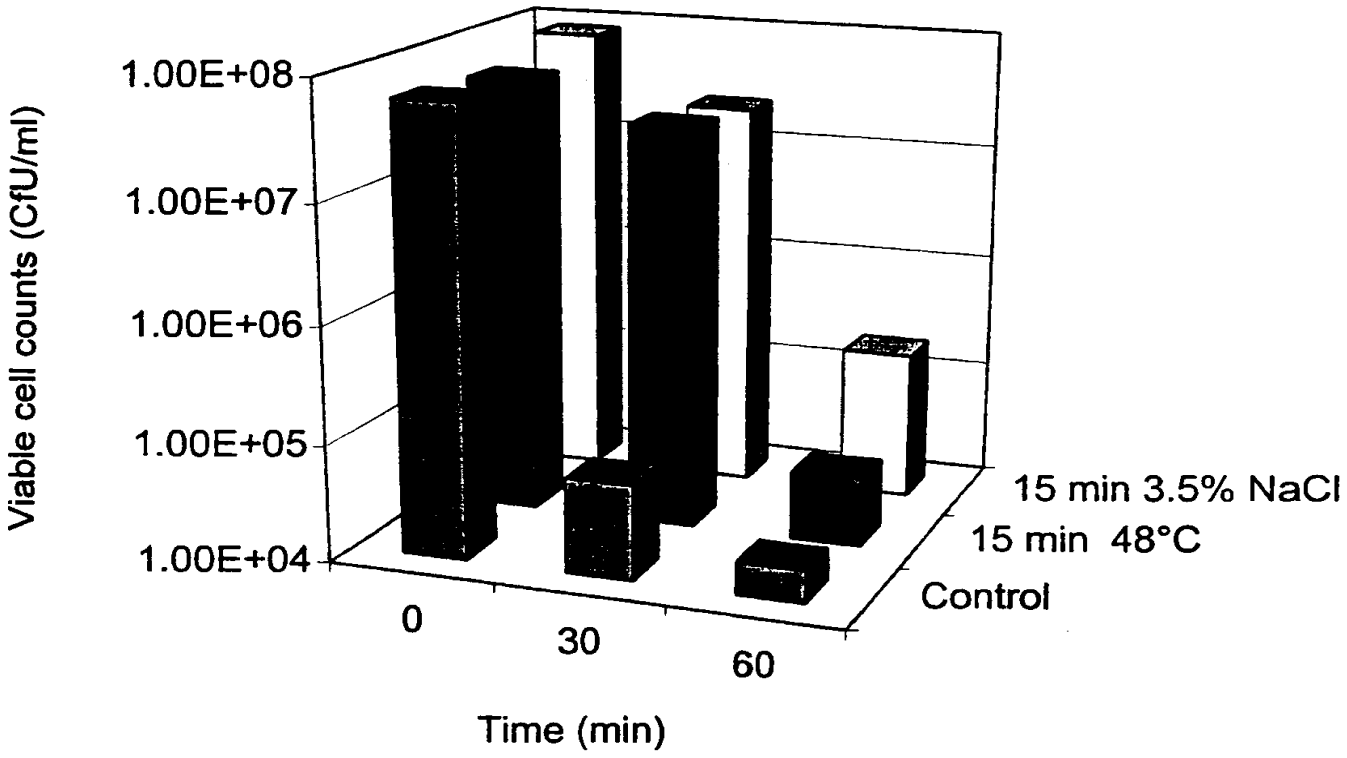

1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Viable cell counts (CfU/ml)
0
30
60
Time (min)
15 min 3.5% NaCl
15 min 48°C
Control

Figure 8

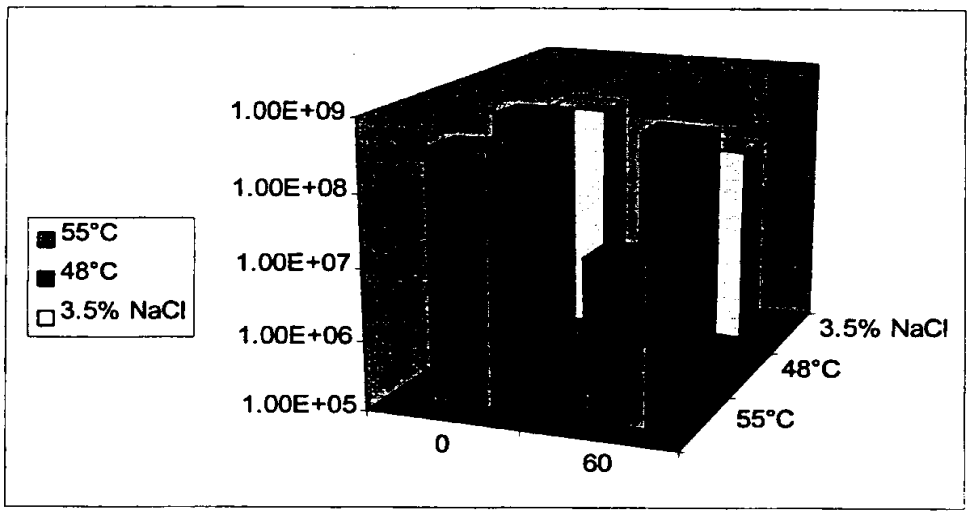

55°C
48°C
3.5% NaCl
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
0
60
3.5% NaCl
48°C
55°C

Figure 9